



## Original Article

# Associations between proteasomal activator PA28 $\gamma$ and outcome of oral squamous cell carcinoma: Evidence from cohort studies and functional analyses



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## SUMMARY

**Background:** PA28 $\gamma$  was suggested to play a role in malignant progression. This paper aimed to investigate the association between PA28 $\gamma$  and the prognosis of oral squamous cell carcinoma (OSCC) in cohort studies.

**Methods:** The PA28 $\gamma$  expression level was assessed by immunohistochemistry in a total of 368 OSCC patients from three independent cohorts. The Cox proportional hazards regression model was used to determine multivariate hazard ratios for Overall Survival (OS). Model discrimination was measured using C Statistic. Additionally, OS was analyzed in Head Neck Squamous Cell Carcinoma (HNSCC) patients from The Cancer Genome Atlas (TCGA) data set. Functional analyses were conducted both in-vitro and in-vivo.

**Findings:** The median follow-up times of patients in the three studies were 60, 52, and 51 months. High expression of PA28 $\gamma$  was identified in tumors from 179 of 368 patients (48.6%). Compared with low expression, high expression of PA28 $\gamma$  was strongly associated with worse OS, with relative risks of 5.14 (95% CI, 2.51–10.5;  $P < 0.001$ ), 2.82 (95% CI, 1.73–4.61;  $P < 0.001$ ), and 3.85 (95% CI, 1.59–9.37;  $P = 0.003$ ). PA28 $\gamma$  expression was also associated with disease-free survival in all three cohorts ( $P < 0.005$ ). These findings are consistent with TCGA HNSCC data ( $P < 0.006$ ). The prediction of all-cause mortality was significantly improved when PA28 $\gamma$  was added to the traditional clinical factors (Model 3, C statistic value: 0.78 VS 0.73,  $P = 0.016$ ). In functional analyses, we found that PA28 $\gamma$  silencing dramatically inhibited the growth, proliferation and mobility of OSCC cells in vitro and reduced tumor growth and angiogenesis in tumor-bearing mice.

**Interpretation:** PA28 $\gamma$  overexpression is associated with adverse prognosis in patients with OSCC. The aberrant expression of PA28 $\gamma$  may contribute to the pathogenesis and progression of OSCC.

**Research in context:** OSCC is one of the most common HNSCC, which have a high lethally rate. However, few prognostic markers have been applied in the clinical practice. We found that PA28 $\gamma$  in OSCC tumor tissues were significantly high expression than those in normal tissues. As the results of the three cohorts from two independent research centers and from an additional validation cohort from a US population in the TCGA dataset, we demonstrate PA28 $\gamma$  is a good predictor of the risk of death in OSCC. Meanwhile, we demonstrate PA28 $\gamma$  have a potential role in OSCC tumorigenesis.

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## 1. Introduction

OSCC is one of the most common HNSCC, with an estimated 260,000 new cases and 120,000 deaths worldwide each year (Jemal et al., 2011). Despite recent advances in diagnosis and treatment, the 5-year survival rate of patients with OSCC is no more than 50% (Panzarella et al., 2014). Over the past two decades, numerous prognostic and predictive markers for clinical outcomes in OSCC have been proposed (Ratajczak-

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Wrona et al., 2013); however, few have been applied in clinical practice due to the non-reproducibility of the initial findings (Choi and Myers, 2008; Principe et al., 2013). To date, the classical clinic pathological parameters of tumor such as primary site, tumor stage, lymph nodal stage and clinical TNM stage remain the most significant factors to affect outcome of patients with OSCC. However, it is impossible to predict patients at a high risk of death mainly based on these parameters. Therefore, it is critical to identify novel and effective prognostic predictors and therapeutic targets for treating this common malignancy.

In eukaryotic cells, proteasomes play an essential role in intracellular proteolysis and are involved in the control of most biological processes through regulated degradation of key proteins. PA28 is a member of a unique family of proteasomal activators that has the ability to stimulate the proteolytic activity of the 20S core proteasome independent of ubiquitination and ATP (Li et al., 2007). Unlike PA28 $\alpha$  and PA28 $\beta$ , PA28 $\gamma$  (also known as Ki antigen, 11S $\gamma$ , or REG $\gamma$ ) localizes in the nucleus and forms a homo-heptamer (Kloetzel and Ossendorp, 2004; Rechsteiner et al., 2000; Rivett and Hearn, 2004). PA28 $\gamma$  is regulated by MEK3, B-RAF, caspase-3/7 and targeted by miR-7-5p, is a multifunctional protein that is involved in the degradation of important regulatory proteins, such as SRC-3, PTTG1 and cyclin-dependent kinase inhibitors p21/16/19 in an ubiquitin- and ATP-independent manner, and has been implicated in the regulation of cell cycle progression (Li et al., 2007; Araya et al., 2002; Chen et al., 2007; Ying et al., 2006; Shi et al., 2015). Moreover, PA28 $\gamma$ -deficient mice have been shown to exhibit growth retardation (Barton et al., 2004). Several targets of PA28 $\gamma$  have been identified in recent years, suggesting that it plays important roles in angiogenesis, hepatic lipid metabolism, infectious diseases and premature aging (Liu et al., 2014; Dong et al., 2013; Yan et al., 2014; Li et al., 2013). PA28 $\gamma$  is over-expressed in some cancer tissues, suggesting that this protein may also have a potential role in tumorigenesis (Wang et al., 2011; Roessler et al., 2006). Some studies found that PA28 $\gamma$  may facilitate the turnover of the tumor suppressor p53 by promoting murine double minute 2 (MDM2)-mediated p53 ubiquitination (He et al., 2012; Zhang and Zhang, 2008) and PA28 $\gamma$  could take part in the ATM-DBC1-SIRT1 axis induced p53-dependent apoptosis (Magni et al.,

2014). Recently researchers found that mutant p53 (p53-R248Q) could up-regulate PA28 $\gamma$  in endometrial cancer (Wang et al., 2015), thus, there is an auto-regulatory feedback loop between p53 and PA28 $\gamma$  (Wan et al., 2014). Nevertheless, the mechanism by which PA28 $\gamma$  exerts its effects on tumor cells remains unclear.

In our previous study, we conducted a comprehensive proteomic analysis to identify candidate biomarkers in OSCC (Wang et al., 2008). Expression levels of 52 proteins in OSCC tumor tissues were significantly different from those in normal tissues (Wang et al., 2009). One of these proteins was PA28 $\gamma$ . The bioprocesses and interaction network analysis indicated that PA28 $\gamma$  might play an important role in malignant transformation. Given these findings, we hypothesized that PA28 $\gamma$  may be involved in malignant development and progression of OSCC and would have effect on the prognosis of this disease. To test this hypothesis, we first explored the protein expression profile of PA28 $\gamma$  and its relations with the outcome of OSCC patients in three independent cohorts from two centers. Then, we constructed models to predict death of patients with OSCC using PA28 $\gamma$  individually and jointly with other prognostic factors identified in these three cohorts. Finally, we investigated the effects of PA28 $\gamma$  on the biological behavior of OSCC cells both in vitro and in vivo.

## 2. Methods and patients

### 2.1. Patients

The Institutional Review Boards of the West China Hospital of Stomatology, Sichuan University and Guangdong Provincial Stomatological Hospital approved this study. The study was approved by the ethics committee both of the West China Hospital of Stomatology and the Guangdong Provincial Stomatological Hospital and was conducted in agreement with the Helsinki Declaration. Written informed consent was provided by all participants at baseline and during follow-up.

A total of 368 postoperative patients with primary OSCC tumors received regular follow-up. Follow-up visits entailed at least a medical history and clinical examination. In addition to scheduled visits, all patients

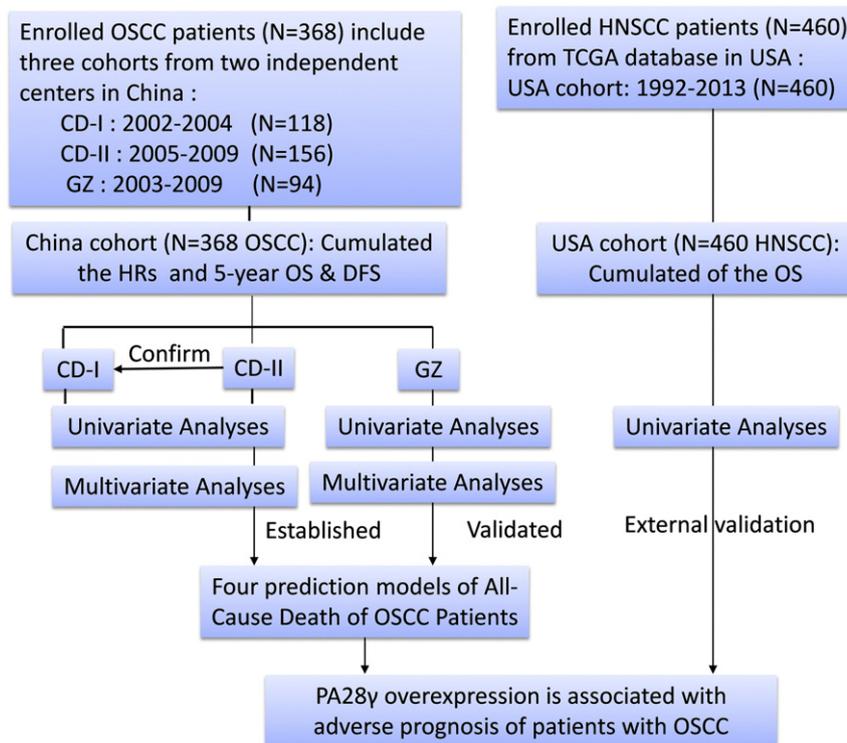


Fig. 1. Study flow chart.

could initiate visits if they were concerned that they had recurrence or a new primary tumor. The survival time of each patient was calculated from the day of surgery until the time of cancer-related death or the end of the follow-up period, death for other reasons led to censoring of data. The detailed information of three cohorts was described in Supplemental Patients and Methods.

An independent cohort of 460 patient specimens obtained between 1992 and 2013 in the TCGA database (<http://tcga-data.nci.nih.gov/tcga/>) was used as an external validation cohort to validate the prognostic value of PA28γ in patients with HNSCC (Table S1).

2.2. Laboratory experiments

All animal studies were approved by the Animal Care and Use Committee, State Key Laboratory of Oral Diseases, in compliance with the Guide for the U.S. Public Health Service's policy on humane care and use of laboratory animals (Kilkenny et al., 2010). Animals were housed within 12-h light/dark cycles and received food, standard rodent chow, and water ad libitum in compliance with the Association for Assessment and Accreditation of Laboratory Animal Care International guidelines. Other methods are detailed in Supplemental Patients and Methods including Cell Culture and siRNA transfections, western blot analysis, MTT, colony-formation, propidium iodide (PI) staining, flow cytometry, TUNEL, cell invasion, cell migration, in vivo tumor-formation assay, and immunohistochemistry.

2.3. Statistical analysis

Baseline characteristics among the patients were compared using the mixed linear model for continuous variables and the  $\chi^2$  test or Fisher's Exact Test for categorical variables. OS and DFS were estimated using the Kaplan–Meier method, with a log-rank test in a univariate analysis. Multivariate survival analysis was done using the Cox proportional hazards model. Model discrimination was measured using C Statistic for survival analysis. Receiver operating characteristic (ROC) curve area was used in the prediction model.

Statistical analyses were performed using SAS software, version 9.3 (SAS Institute Inc., Cary, NC). Unless stated otherwise, two-sided  $P < 0.05$  were considered significant. Details on data analysis are provided in the Supplementary.

3. Results

3.1. Patient and disease characteristics

A total of 368 patients from three independent cohorts (118, 156, and 94 patients in CD-I cohort, CD-II cohort, and GZ cohort, respectively) were included in this study (Fig. 1). All patients were treated with curative intent. Some of these patients had been treated by radiotherapy and/or chemotherapy. The mean age and gender distribution were comparable across the three cohorts. The median durations of follow-up in the cohorts were 60, 52, and 51 months, respectively. IHC staining showed that PA28γ has a very clear nuclear positive in most tumor but not normal tissues (Fig. S1). High expression of PA28γ was observed on OSCC cell lines, and in 52.54%, 38.46%, and 60.64% of the patients in the CD-I cohort, CD-II cohort and GZ cohort, respectively (Table 1 and Fig. S2). This finding is consistent with the TCGA database analysis of PA28γ mRNA abundance in human primary oral cancers (Fig. S3a).

3.2. Univariate and multivariate analyses of PA28γ expression and its predictive value

In all three cohorts, the results of univariate analysis showed that OS at five years was associated with PA28γ expression (Table 2). For the CD-I cohort, estimated 5-year OS values for patients in the low and high PA28γ expression groups were 84% (95% CI, 0.71–0.91) and 40%

(95% CI, 0.28–0.52; Fig. 2a), respectively. For the CD-II cohort, the estimated 5-year OS values for patients in the low and high PA28γ expression groups were 67% (95% CI, 0.56–0.57) and 28% (95% CI, 0.18–0.40; Fig. 2b), respectively. For the joint CD-I and CD-II cohorts, the estimated 5-year OS for patients also showed that the risk increased associated with positive staining ( $P < 0.001$ ; Fig. 2c). For the GD validation cohort, the estimated 5-year OS values for patients in the low and high PA28γ expression groups were 89% (95% CI, 0.74–0.96) and 57% (95% CI, 0.43–0.69; Fig. 2d), respectively. Consistent with an external validation cohort of 460 HNSCC patients from the TCGA database analysis in the US population, high PA28γ mRNA was associated with poor survival ( $P = 0.016$ , Fig. S3b). Furthermore, the association between PA28γ high

Table 1 Baseline characteristics of the patients with oral squamous cell carcinoma in three cohorts.

Characteristic	CD-I cohort	CD-II cohort	GZ cohort	P value*
	(N = 118)	(N = 156)	(N = 94)	
	Number (%)	Number (%)	Number (%)	
Age-yr (mean ± sd)	58.97 ± 13.67	58.76 ± 10.62	60.39 ± 12.35	0.563
<60 yr	55 (46.61)	76 (48.72)	42 (44.68)	0.821
≥60 yr	63 (53.39)	80 (51.28)	52 (55.32)	
Sex				
Male	81 (68.64)	112 (71.79)	55 (58.51)	0.089
Female	37 (31.36)	44 (28.21)	39 (41.49)	
Smoking				
Never	65 (55.08)	76 (48.72)	50 (53.19)	0.556
Ever	53 (44.92)	80 (51.28)	44 (46.81)	
Drinking				
Never	62 (52.54)	76 (48.72)	55 (58.51)	0.324
Ever	56 (47.46)	80 (51.28)	39 (41.49)	
Differentiation				
High	75 (63.56)	104 (66.67)	72 (76.60)	0.144
Moderate	37 (31.36)	40 (25.64)	20 (21.28)	
Low	6 (5.08)	12 (7.69)	2 (2.13)	
Primary site				
Ventral tongue/floor of mouth	50 (42.37)	71 (45.51)	54 (57.45)	<0.001
Buccal mucosa	18 (15.25)	27 (17.31)	10 (10.64)	
Gingiva	15 (12.71)	23 (14.74)	25 (26.60)	
Others <sup>a</sup>	35 (29.66)	35 (22.44)	5 (5.32)	
Tumor stage				
T1	36 (30.51)	34 (21.79)	19 (20.21)	<0.001
T2	61 (51.69)	48 (30.77)	48 (51.06)	
T3	15 (12.71)	53 (33.97)	12 (12.77)	
T4	6 (5.08)	21 (13.46)	15 (15.96)	
Nodal stage				
N0	82 (69.49)	108 (69.23)	71 (75.53)	0.522
N1–N3	36 (30.51)	48 (30.77)	23 (24.47)	
Clinical TNM stage				
I	28 (23.73)	29 (18.59)	15 (15.93)	0.009
II	45 (38.14)	37 (23.72)	38 (40.43)	
III	24 (20.34)	57 (36.54)	20 (21.28)	
IV	21 (17.80)	33 (21.15)	21 (22.34)	
Surgery type				
Local	49 (41.53)	55 (35.26)	18 (19.15)	<0.001
Unilateral neck	62 (52.54)	82 (52.56)	65 (69.15)	
Bilateral neck	6 (5.08)	4 (2.56)	6 (5.32)	
Other	1 (0.85)	15 (9.62)	5 (5.32)	
Radiotherapy				
Yes	9 (7.63)	28 (17.95)	15 (15.96)	0.044
No	109 (92.37)	128 (82.05)	79 (84.04)	
Chemotherapy				
Yes	65 (55.08)	84 (53.85)	42 (44.68)	0.262
No	53 (44.92)	72 (46.15)	52 (55.32)	
Radiotherapy or chemotherapy				
Yes	67 (56.78)	93 (59.62)	49 (52.13)	0.512
No	51 (43.22)	63 (40.38)	45 (47.87)	
PA28γ				
Low expression	56 (47.46)	96 (61.54)	37 (39.36)	0.002
High expression	62 (52.54)	60 (38.46)	57 (60.64)	

Abbreviations: CD, Chengdu; GZ, Guangzhou.  
 \* P value of comparison between studies was generated using mixed linear model for continuous variables and chi-square test or Fisher's exact test for categorical variables.  
<sup>a</sup> Others included hard palate, mandibular and lip mucosa.

expression with lower rates of 5-year Disease-Free Survival (DFS) was also statistically significant in those three independent cohorts ( $P < 0.001$ ; CD-II:  $P < 0.001$ ; joint CD-I and -II cohorts:  $P < 0.001$ ; GD:  $P = 0.004$ ; Table S2; Fig. S4). Several conventional prognostic factors, including lower cell differentiation, positive nodal stage, higher tumor stage, higher clinical TNM stage and radiotherapy or chemotherapy, were associated with a significantly increased risk of death. History of smoking and alcohol consumption were significantly related with survival ( $P < 0.005$ ). Some of these factors were included in the multivariable Cox proportional-hazards model and fixed.

Results of the predictive analysis are provided in Table 3. Strong PA28 $\gamma$  expression was independently associated with significantly reduced OSCC patients' OS in the CD-I cohort (HR, 5.14; 95% CI, 2.51–10.5;  $P < 0.001$ ) after accounting for smoking history, drinking history, cell differentiation, tumor stage, nodal stage and radiotherapy or chemotherapy; this was later confirmed in the CD-II cohort (HR, 2.82; 95% CI, 1.73–4.61;  $P < 0.001$ ). There is evidence that patients with PA28 $\gamma$  high expression had worse DFS in the CD-I and CD-II cohorts, with hazard ratios of 3.82 (95% CI, 2.12–6.88;  $P < 0.001$ ) and 2.96 (95% CI, 1.87–4.69;  $P < 0.001$ ), respectively (Table S3), after accounting for

the same factors as in the OS analysis. Furthermore, in the joint CD-I and CD-II cohorts and in the validation GZ cohort, the analysis results showed similar patterns ( $P < 0.001$ ; Table 3; Table S3).

### 3.3. Prediction models for all-cause death of OSCC patients

Multivariable models were constructed for the prediction of all-cause death in OSCC patients by using the combined CD cohort as the discovery cohort, and the GD cohort as a validation cohort. We assessed model discrimination using the C statistic for predictive value and compared the difference between basic models and models including PA28 $\gamma$  expression (Table S4). For the discovery of CD cohort, in Model 1, among the basic risk factors, the C statistic value of PA28 $\gamma$  was highest. The C statistic increased significantly when PA28 $\gamma$  was combined with those conventional risk factors in Models 2 to 4. In Models 3 and 4, when PA28 $\gamma$  was added, the C statistic was larger than 0.75, above which the prediction model is considered relatively good. Similar results were found in the validation GZ cohort. ROC curves were constructed for the mode Models 3 and 4, in which the area under the ROC curve indicates the C statistic (Figs. 3, S5).

**Table 2**

Univariate analyses of selected characteristics with survival among patients with oral squamous cell carcinoma.

Characteristic	CD-I cohort (N = 118)		CD-II cohort (N = 156)		GZ cohort (N = 94)	
	OS at five years	P value*	OS at five years	P value*	OS at five years	P Value*
	(95% CI)		(95% CI)		(95% CI)	
Age						
<60 yr	0.65 (0.51–0.76)	0.510	0.47 (0.36–0.58)	0.139	0.81 (0.66–0.90)	0.057
≥60 yr	0.56 (0.43–0.68)		0.56 (0.45–0.66)		0.61 (0.46–0.73)	
Sex						
Male	0.58 (0.46–0.67)	0.327	0.54 (0.45–0.63)	0.422	0.67 (0.52–0.78)	0.352
Female	0.68 (0.50–0.80)		0.45 (0.30–0.59)		0.74 (0.58–0.85)	
Smoking history						
Never	0.75 (0.63–0.84)	<0.001	0.58 (0.46–0.68)	0.108	0.75 (0.60–0.85)	0.097
Ever	0.43 (0.30–0.56)		0.46 (0.35–0.57)		0.64 (0.48–0.76)	
Drinking history						
Never	0.69 (0.56–0.79)	0.037	0.65 (0.54–0.75)	<0.001	0.78 (0.65–0.87)	0.027
Ever	0.52 (0.38–0.64)		0.39 (0.28–0.49)		0.58 (0.41–0.72)	
Cell differentiation						
High	0.70 (0.58–0.79)	0.003	0.58 (0.48–0.66)	0.020	0.75 (0.63–0.83)	0.042
Moderate or Low	0.44 (0.29–0.58)		0.40 (0.27–0.53)		0.55 (0.32–0.72)	
Primary site						
Ventral tongue/floor of mouth	0.59 (0.44–0.72)	0.814	0.56 (0.44–0.67)	0.477	0.72 (0.58–0.82)	0.265
Buccal mucosa	0.67 (0.40–0.83)		0.44 (0.25–0.62)		0.86 (0.33–0.98)	
Gingiva	0.67 (0.38–0.85)		0.43 (0.23–0.62)		0.50 (0.25–0.71)	
Others <sup>a</sup>	0.57 (0.39–0.71)		0.54 (0.37–0.69)		0.60 (0.13–0.88)	
Tumor stage						
T1 or T2	0.66 (0.55–0.74)	0.003	0.56 (0.45–0.66)	0.183	0.76 (0.64–0.85)	0.076
T3 or T4	0.33 (0.15–0.53)		0.47 (0.36–0.58)		0.53 (0.32–0.71)	
Nodal stage						
N0	0.72 (0.60–0.80)	<0.001	0.61 (0.51–0.70)	<0.001	0.78 (0.67–0.86)	<0.001
N1–N3	0.36 (0.21–0.51)		0.31 (0.19–0.44)		0.43 (0.23–0.62)	
Clinical TNM stage						
I or II	0.76 (0.65–0.85)	<0.001	0.61 (0.48–0.71)	0.024	0.79 (0.66–0.88)	0.041
III or IV	0.36 (0.22–0.49)		0.46 (0.35–0.55)		0.57 (0.40–0.71)	
Surgical method						
Local	0.61 (0.46–0.73)	0.878	0.60 (0.46–0.72)	0.116	0.78 (0.51–0.91)	0.318
Unilateral or bilateral or other	0.60 (0.48–0.71)		0.48 (0.38–0.57)		0.68 (0.56–0.77)	
Radiotherapy						
Yes	0.78 (0.36–0.94)	0.347	0.43 (0.25–0.60)	0.218	1.00 (1.00–1.00)	0.008
No	0.59 (0.48–0.68)		0.54 (0.45–0.62)		0.64 (0.52–0.74)	
Chemotherapy						
Yes	0.74 (0.61–0.83)	0.002	0.57 (0.46–0.67)	0.204	0.75 (0.58–0.86)	0.147
No	0.44 (0.31–0.57)		0.46 (0.34–0.57)		0.65 (0.51–0.77)	
Radiotherapy or chemotherapy						
Yes	0.75 (0.62–0.83)	<0.001	0.58 (0.47–0.67)	0.097	0.78 (0.63–0.88)	0.015
No	0.42 (0.28–0.55)		0.43 (0.31–0.55)		0.60 (0.44–0.73)	
PA28 $\gamma$						
Low expression	0.84 (0.71–0.91)	<0.001	0.67 (0.56–0.75)	<0.001	0.89 (0.74–0.96)	<0.001
High expression	0.40 (0.28–0.52)		0.28 (0.18–0.40)		0.57 (0.43–0.69)	

Abbreviations: OS, Overall Survival; CD, Chengdu; GZ, Guangzhou.

\* P value was determined using the log-rank test.

<sup>a</sup> Others included hard palate, mandibular and lip mucosa.

3.4. Validation the functional role of PA28γ both in vitro and in vivo

On the basis of the clinical findings described above, we evaluated the effect of PA28γ on OSCC cell lines and xenograft models. We hypothesized that PA28γ might act as a tumor promoter. If so, PA28γ silencing should reverse some of the early processes of tumorigenesis. PA28γ silencing in both OSCC cell lines caused decreased cell viability and colony growth (Figs. S6, 4a). However, this silencing had no such effect on HOK cells. A significant induction of apoptosis was observed after treatment of cells with PA28γ-specific siRNA (Fig. 4b). Similar results were also observed in the TUNEL assay (Fig. S7). These results suggest that PA28γ silencing can inhibit cell proliferation via induction of apoptosis in OSCC cells. Moreover, our data suggested that PA28γ silencing could inhibit the migration and invasion of OSCC cells in vitro (Fig. S8).

We further investigated whether PA28γ silencing could inhibit tumor growth in vivo. OSCC cells treated with PA28γ siRNA modified with 2' OMe and 3' Chol (PA28γ-si group) which have a high effective interference (Fig. 4c), scramble siRNA (NS-si group) or PBS (CTRL group) were transplanted subcutaneously on the right back of BALB/c nude mice. Tumor growth in the PA28γ-si group was much slower than the other two groups. There was a 40–50% reduction in the average tumor volume in the PA28γ-si group (Fig. 4d, e). To investigate the potential mechanisms underlying the effects of PA28γ silencing in vivo, we examined tumor cell proliferation, microvessel density (MVD) (Rechsteiner and Hill, 2005), and tumor cell apoptosis. As shown in Fig. S9, dramatic reductions in PCNA expression and tumor angiogenesis and significant increases in TUNEL-positive nuclei were found in the tumors in the PA28γ-si group compared with those in the other two groups.

4. Discussion

In the current study, our results showed that the proteasomal activator PA28γ is a prognostic biomarker in OSCC with higher expression levels correlating with worse outcomes compared with normal tissues. We studied three cohorts from China and found consistent results supporting a pronounced effect of PA28γ as a prognostic biomarker with a total of 368 patients, which was confirmed by an external validation cohort of 460 HNSCC patient specimens from the TCGA database. The corresponding role of this gene in regulating tumorigenesis and metastasis was also evaluated.

PA28γ is a member of the PA28 protein family, which has been shown to bind specifically to 20S proteasomes and stimulate the hydrolysis of peptides (Rechsteiner et al., 2000). The PA28γ–20S proteasome pathway plays a very important role in cellular processes. Two recent studies have indicated a role for PA28γ in the regulation of the cell cycle and cell proliferation (Barton et al., 2004; Li et al., 2009). Some cellular targets of PA28γ related to the regulation of cell apoptosis have also been identified (He et al., 2012; Liu et al., 2010). PA28γ is overexpressed in some types of cancers and has been linked with multiple cancer-related pathways (He et al., 2012). Our results indicated that PA28γ may be a tumor promoter gene that can contribute to the development of a more aggressive form of oral carcinoma. Its expression negatively correlates with patient survival. An auto-regulatory feedback loop has recently been reported between p53 and PA28γ, while a p53 mutation, which is the most comprehensive genomic characterization of HNSCC (Cancer Genome Atlas N, 2015), could enhance PA28γ transcription in some cancer cells (Ali et al., 2013). Therefore, the prognostic significance of PA28γ may be driven by p53 mutation; this mechanism warrants further investigation.

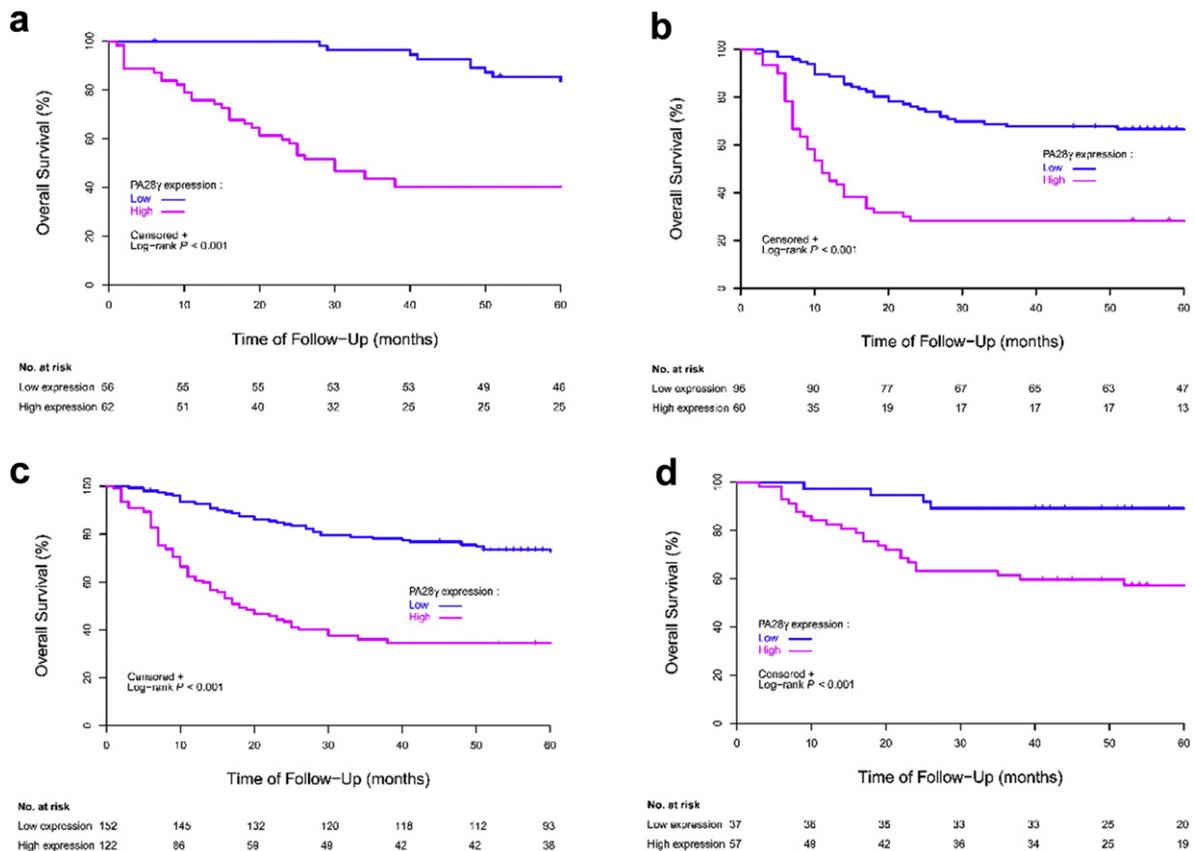


Fig. 2. Overall Survival (OS) of OSCC patients with high and low expression of PA28γ in Three cohorts defined by the Kaplan–Meier survival curves. (a) Overall Survival in CD-I cohort. (b) Overall Survival in CD-II cohort. (c) Overall Survival in CD-I and -II cohorts. (d) Overall Survival in GZ cohort.

**Table 3**  
Multivariate analyses of survival among patients with oral squamous cell carcinoma.

Characteristic	CD-I cohort (N = 118)		CD-II cohort 2 (N = 156)		CD cohort combined (N = 274)		GZ cohort (N = 94)	
	OS at five years		OS at five years		OS at five years		OS at five years	
	HR (95% CI)	P Value*	HR (95% CI)	P Value*	HR (95% CI)	P Value*	HR (95% CI)	P Value*
Smoking history								
Never	Reference	0.072	Reference	0.979	Reference	0.180	Reference	0.349
Ever	1.87 (0.65–3.68)		1.01 (0.59–1.72)		1.32 (0.88–1.98)		1.45 (0.67–3.13)	
Drinking history								
Never	Reference	0.717	Reference	0.023	Reference	0.065	Reference	0.103
Ever	1.14 (0.57–2.28)		1.86 (1.09–3.16)		1.47 (0.98–2.22)		1.89 (0.88–4.07)	
Cell differentiation								
High	Reference	0.036	Reference	0.078	Reference	0.006	Reference	0.014
Moderate or Low	1.98 (1.05–3.75)		1.55 (0.95–2.50)		1.70 (1.16–2.48)		2.84 (1.23–6.52)	
Tumor stage								
T1 or T2	Reference	0.040	Reference	0.399	Reference	0.011	Reference	0.991
T3 or T4	2.20 (1.04–4.69)		1.23 (0.76–1.97)		1.65 (1.12–2.44)		1.01 (0.46–2.19)	
Nodal stage								
N0	Reference	0.316	Reference	0.012	Reference	0.016	Reference	0.016
N1–N3	1.40 (0.72–2.72)		1.89 (1.15–3.10)		1.62 (1.09–2.40)		2.61 (1.20–5.69)	
Radiotherapy or chemotherapy								
Yes	Reference	0.004	Reference	0.141	Reference	0.001	Reference	0.051
No	2.47 (1.34–4.53)		1.44 (0.89–2.35)		1.82 (1.26–2.61)		2.17 (1.00–4.69)	
PA28 $\gamma$								
Low expression	Reference	<0.001	Reference	<0.001	Reference	<0.001	Reference	<0.001
High expression	5.14 (2.51–10.5)		2.82 (1.73–4.61)		3.02 (1.05–4.43)		6.39 (2.12–19.3)	

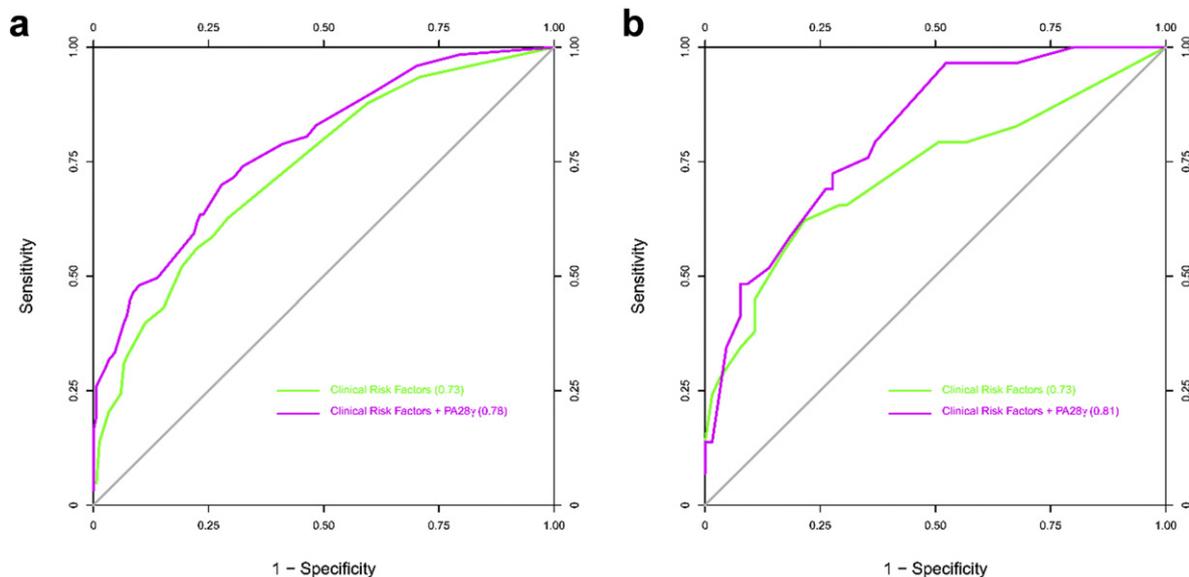
Abbreviations: OS, Overall Survival; CD, Chengdu; GZ, Guangzhou; HR, Hazard Ratio.

\* P value was determined using Cox proportional-hazards model.

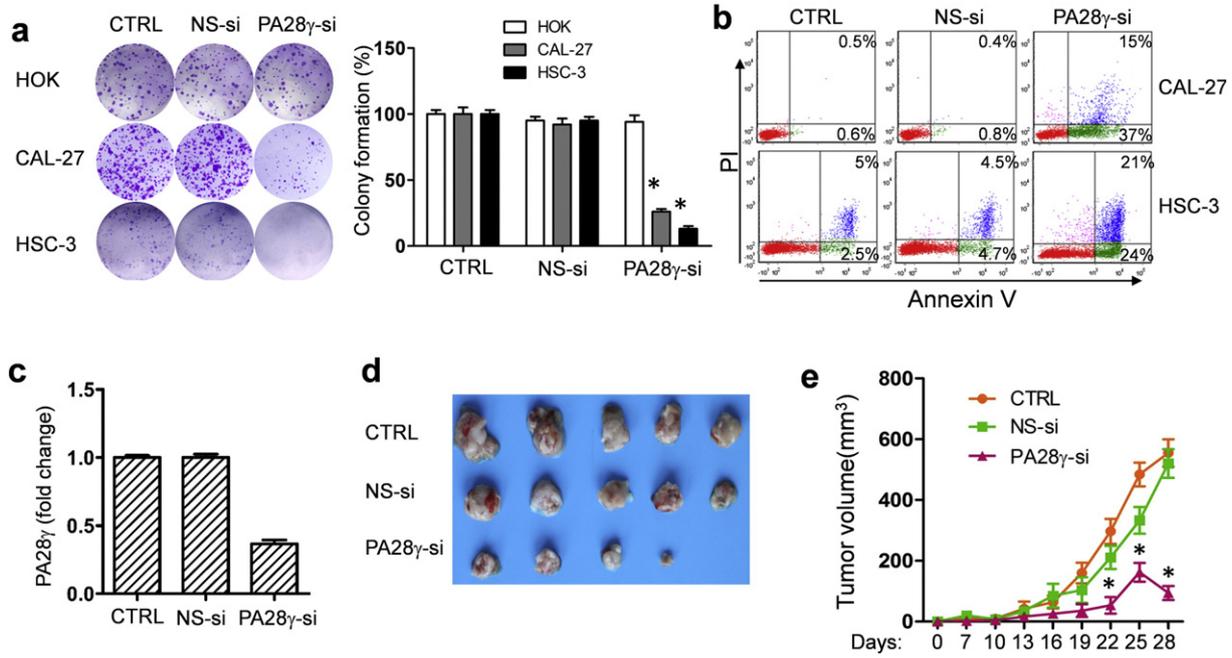
We explored the PA28 $\gamma$  mRNA (gene name: PSME3) expression level in the head and neck cancer group from the TCGA database. Very few genomic changes (2 cases of mutation and 1 case of amplification in 279 head and neck squamous cell carcinoma cases) were observed, suggesting that the PA28 $\gamma$  contribution to oral cancer may not be through genomic events, but more likely through some local condition change events. This hypothesis is under further study in our laboratory. To date, there has been no well-established predictive model for all-cause death of patients with OSCC, or even head and neck cancer. In this study, we generated a series of basic models that included four conventional risk factors and one candidate biomarker, PA28 $\gamma$ . The integrated discrimination improvement was estimated when PA28 $\gamma$  was incorporated into different combinations of established risk factors in Models 2, 3, and 4. The incorporation of PA28 $\gamma$  with established risk

factors improved the risk prediction for death, as shown by a substantial increase in the C Statistic. We also used an alternative model by replacement of tumor and nodal stage with clinical TNM stage in Model 3, as the latter is a more commonly used clinical prognosis factor. Thus, the role of each factor independently and in combination in predicting all-cause death could be determined. Most importantly, these models highlight the prognostic value of PA28 $\gamma$  by itself or in combination with conventional predictors. Our data were notable in that the replacement of multiple clinical factors with a simplified alternative clinical factor yields consistent results that would be extended to the clinical setting.

Furthermore, to evaluate the molecular basis for the clinical association described above, we investigated the biologic role of PA28 $\gamma$  in cancer cell lines and OSCC xenograft models. Our study provided the first biological evidence for the role of PA28 $\gamma$  in tumor growth and



**Fig. 3.** ROC curves for all-cause death of OSCC patients with or without PA28 $\gamma$  expression in three cohorts of two independent centers. (a) ROC curve for Model 3 in CD-I and -II Cohorts. (b) ROC curve for Model 3 in GZ cohort.



**Fig. 4.** Functional role of PA28γ on the tumor growth. (a) Colony formation assay. PA28γ silencing led to colony formation inhibition in two OSCC cell lines (HSC-3 and CAL-27), after 10 days transfection with siRNA. All data are presented as the mean ± SD of three independent experiments (\**P* < 0.05). (b) PA28γ silencing induced the apoptosis in OSCC cells, determined by Flow Cytometry assay, after 48 h transfection with siRNA. All data are presented as the mean ± SD of three independent experiments (\**P* < 0.05). (c) Chemically-modified PA28γ-si could inhibit PA28γ gene expression in HSC-3, illustrated by qPCR, 48 h. All data are presented as the mean ± SD of three independent experiments (\**P* < 0.05) (d and e) The tumor volumes (mean ± SD) of PA28γ-si group were significantly smaller compared to the other two groups, 30 days after injecting transfected cells. (n = 5, \**P* < 0.05).

metastasis. Beyond its significance in metastasis-related outcome prediction, our data also showed that PA28γ silencing significantly suppressed tumor angiogenesis. Further studies are in progress in our laboratory.

The clinically significant role of PA28γ expression as a surrogate marker in OSCC is clearly established in this study. However, our model does have some limitations. Some reports have described prognosis according to a primary sub-site, which differs in HNSCC (Chung et al., 2014), the main primary sites of patients with OSCC in our cohorts were ventral tongue or floor of mouth. Although there is no difference between sub-sites in our cohorts, we could not examine survival outcomes based on PA28γ status and primary site, given the limited number of subset cases. Therefore, our data show that the use of PA28γ as a prognostic biomarker in OSCC requires more investigation before broad application in the clinical setting.

**5. Conclusion**

Overall, we found that PA28γ is a good predictor of the risk of death in OSCC and adds additional information to well-established prognostic factors, which were derived from OS and DFS analyses as well as the eventual four statistic models in those cohorts. Meanwhile, the functional studies in vitro and in vivo in a mouse xenograft model also validated the cellular effects of PA28γ in OSCC. However, the molecular mechanisms responsible for its function are still unclear. The identification of the targets and action model of PA28γ in OSCC needs to be further delineated in our further study.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2015.07.004>.

**Contributors**

Q. C. Z. W directed this study, coordinated the research. Q. C. Z. W, and J. L wrote the final manuscript. J. L, X. F, C. S and X. Z have contributed to the design of the experiments and performed research. M. Z, L. L, G. Liao, N. G, Y. L, X. L and L. Y have contributed to provide the study

materials or patients. J. L, L. X, X. X, H. X, R. W, D. Z, H. D, Y. Z, P. D, Y. W, Z. W, N. J, G. Luo and L. J have contributed to the collection and assembly of data. J. L, Z. W, X. Z, H. X and T. L. have contributed to data analysis and interpretation. All authors reviewed and revised the paper.

**Conflict of interests**

The authors declare no potential conflicts of interest.

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